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PCR-Based Retrospective Evaluation of Diagnostic Samples for Emergence of Porcine Deltacoronavirus in US Swine

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Highlights

- 1,734 clinical samples collected between Oct 2012 and Dec 2013 were tested.
- PDCoV RNA was first detected in a sample from Minnesota collected on Aug 19, 2013.
- PDCoV has been present in US swine at least since Aug 2013.

Abstract

Porcine deltacoronavirus (PDCoV) was first identified in Hong Kong in a regional surveillance study for coronaviruses in 2012 and was detected for the first time in United States (US) swine in February 2014. However, it remains unknown if PDCoV had been introduced into the US prior to that time period. In the present study, 1,734 clinical samples (903 cases) submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for enteric disease diagnosis between October 2012 and December 2013 were tested retrospectively for PDCoV using a virus-specific real-time reverse transcription (RT) PCR targeting conserved region of the membrane gene. PDCoV genome was first detected in a fecal sample collected on August 19th 2013 from Minnesota. Subsequently, PDCoV was observed in samples collected on August 20th and August 27th from Iowa and on August 29th from Illinois. Therefore, with available samples submitted to the ISU VDL, it can be inferred that PDCoV has been present in US swine at least since August 2013.

Keywords

Porcine Deltacoronavirus, PDCoV, retrospective, RT-PCR

1. Introduction

Coronaviruses belong to the order *Nidovirales*, family *Coronaviridae* and subfamily *Coronavirinae* and until recently, were subdivided into 3 genera including *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus* (Carstens, 2010). In 2012, researchers in Hong Kong conducted a regional surveillance study on a variety of domestic and wild animal species for coronaviruses from 2007 through 2011 and identified coronaviruses in birds and mammals that formed a new genus subsequently named *Deltacoronavirus* (Woo et al., 2012; King, 2012). According to that study, porcine deltacoronavirus (PDCoV) has been present in pigs in Hong Kong since 2009. PDCoV is a single stranded, positive sense, enveloped RNA virus with a genome size varying between 25.4 and 26.6 Kb (Woo et al., 2012; Li et al., 2014).

The presence of PDCoV in the US was first announced by the Ohio Department of Agriculture in early February 2014 after which its genetic characterization was reported by several laboratories (Wang et al., 2014; Li et al., 2014; Marthaler et al., 2014). As of April 23, 2015, PDCoV has been detected in 19 US states according to the weekly report on PDCoV in the USDA/APHIS website (www.aphis.usda.gov/animal-health/secd). Nonetheless, it remains unknown if PDCoV had been introduced into the US earlier than 2014 or if it was introduced into the US at the same time as porcine epidemic diarrhea virus (PEDV) as early as April, 2013 (Stevenson *et al.*, 2013). In the present study, we performed retrospective PCR testing on samples which were submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) between October 2012 and December 2013 for investigation of enteric diseases, to determine the earliest date PDCoV was detected in US swine.

2. Materials and Methods

2.1. Clinical Samples

A total of 1,734 clinical samples, including feces, fecal swabs, oral fluids, and environmental and intestinal specimens from cases of porcine diarrhea submitted to the ISU VDL from October 2012 to December 2013, were used in the study. Intestinal samples were fixed in formalin and then embedded in paraffin blocks (FFPE samples) at the time of receipt. FFPE blocks were stored at room temperature and all other specimens were preserved at -80^0 C from the time of original receipt and testing until retrospective testing for PDCoV.

2.2. Sample Processing and Extraction

A small amount of feces, fecal swabs and environmental specimens (swabs/swiffers) were placed in 1 ml PBS and agitated manually for three seconds prior to the extraction step. Viral RNA extraction was conducted with 100 µl of processed samples using the MagMAX^{TM} viral RNA isolation kit (Life Technologies, Carlsbad, CA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA) following manufacturer's instructions. The viral RNA was eluted into 90 µl of elution buffer. Viral RNA was extracted from the FFPE intestinal samples using the MagMAX^{TM} FFPE Total nucleic acid isolation kit (Life Technologies, Carlsbad, CA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA) using manufacturer's recommendations. Viral RNA was eluted into 70 µl of elution buffer. FFPE intestinal tissues from PDCoV positive cases submitted to the ISU VDL and PDCoV positive amplification control (PAC) were included as positive controls.

2.3. PDCoV Real-time RT-PCR

The primers and probe were designed to target the conserved regions of the PDCoV membrane protein gene based on the nucleotide sequences of strains HKU15-44 and HKU15-155 deposited in GenBank (JQ065042.1 and JQ065043.1, respectively). (Woo et al, 2012). Primer and probe sequences were later verified as appropriate for the US virus after sequence information was available. The forward primer sequence was 5'-CGA CCA CAT GGC TCC AAT TC-3', the reverse primer sequence was 5'-CAG CTC TTG CCC ATG TAG CTT-3', and the probe sequence was 5'-CAC ACC AGT CGT TAA GCA TGG CAA GC-3'. The probe was labeled using the FAM/ZEN/3'Iowa Black detector (Integrated DNA Technologies, Coralville, IA, USA). Real-time RT-PCR was conducted on nucleic acid extracts using a Path-ID Multiplex One-Step RT-PCR kit (Life Technologies, Carlsbad, CA). Real-time RT-PCR was performed on an ABI 7500 Fast instrument (Life Technologies, Carlsbad, CA) with the following conditions: 1 cycle of 45°C for 10 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 45 sec.

3. Results

A retrospective study, described here, was conducted to determine if PDCoV was introduced at approximately the same time as PEDV or if this may have occurred independently. The 1,734 clinical samples tested for PDCoV represents 903 ISU VDL cases from eighteen states submitted from early October 2012 to December 2013 to detect various enteric pathogens. A total of five samples tested positive for the presence of PDCoV RNA with C_T values of 14.6, 15.1, 28.1, 36.6 and 36.7, respectively. The states, pathogens and specimens tested from the 903 cases are given

as a total and percentage in Table 1. The numbers of PDCoV positive cases are also included in Table 1.

The PDCoV genome was first detected in a fecal sample collected on August 19th 2013 from a pig in Minnesota with a history of diarrhea and positive for *Salmonella krefeld* but negative for PEDV by PCR followed by detection of PDCoV in another fecal sample collected on August 20th from a pig in Iowa with lesions of ileitis and positive for *Lawsonia intracellularis* but negative for PEDV by PCR. RNA extracted from a fixed intestine sample collected on August 27th 2013 from Iowa that was negative for PEDV and positive for Rotavirus group A, group B and group C also tested positive for PDCoV. Thereafter, PDCoV was detected in fecal samples collected on August 29th 2013 in two additional pigs from Illinois which were negative by PCR for PEDV, transmissible gastroenteritis virus (TGEV) and *Lawsonia intracellularis* and also negative by immunohistochemistry for PEDV, group A rotavirus and TGEV.

4. Discussion

A study conducted to evaluate the presence of *Deltacoronavirus* identified a novel porcine deltacoronavirus genome in swine reported as the HKU15 (strains HKU15-44 and HKU15-155) in Hong Kong, China (Woo et al., 2012). In early February 2014, PDCoV was detected and announced in the US by the Ohio Department of Agriculture. Soon thereafter, genome sequencing and analysis of the PDCoV strains present in the US revealed approximately 99% nucleotide similarity of these newly emerged strains to the porcine deltacoronavirus HKU15-44 and HKU15-155 from China (Wang et al., 2014; Li et al., 2014; Marthaler et al., 2014). It was unknown when PDCoV might have been introduced into the US considering an additional coronavirus (PEDV) had recently been detected in US swine in less than one year of detecting

PDCoV (Stevenson et al., 2013). However, from the present study and with available samples submitted to the ISU VDL at the present time, it can be inferred that PDCoV has been present in US swine at least since August 2013, suggesting that the PDCoV introduction may have been independent of PEDV introduction into the US. Although the origin of PDCoV remains unknown at this time, detection of the deltacoronavirus after PEDV may suggest the potential of additional trans-boundary or foreign animal diseases to enter the US in the future.

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Table

Table1. Categorization of specimens submitted to ISU VDL between 2012 and 2013 for diagnosis of porcine enteric disease and tested in this study for PDCoV RNA.

Classifications	Cases, No. (%)	Positive Cases
State		
Iowa	488 (54.0)	2
North Carolina	110 (12.2)	0
Illinois	57 (6.3)	2
Oklahoma	46 (5.1)	0
Ohio	42 (4.6)	0
Indiana	38 (4.2)	0
Minnesota	36 (3.9)	1
North Dakota	27 (2.9)	0
Nebraska	14 (1.5)	0
Pennsylvania	12 (1.3)	0
Missouri	8 (0.9)	0
Utah	6 (0.6)	0
Wisconsin	6 (0.6)	0
Texas	5 (0.5)	0
Arizona	4 (0.4)	0
South Dakota	2 (0.2)	0
Kansas	1 (0.1)	0
Virginia	1 (0.1)	0

Sample types	
Feces	784 (86.8)
Fecal swab	248 (27.5)
Intestine	49 (5.4)
Oral fluid	26 (2.9)
Environmental	19 (2.1)
Pathogens tested	
TGEV	634 (70.2)
Rotavirus	423 (46.8)
E. Coli	209 (23.1)
Lawsonia	137 (15.2)
PEDV	125 (13.8)
PRCV	30 (3.3)

Note: TGEV – Transmissible gastroenteritis virus

PEDV – Porcine epidemic diarrhea virus

PRCV – Porcine respiratory coronavirus